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We investigated functional interaction of Chk2 and Kiaa0170 (renamed as MDC1). We found that MDC1 interacts with Chk2 in a DNA-damage dependent manner and colocalizes with Chk2 at the sites of DNA damage. Furthermore, we found that the FHA domain of MDC1 and the phosphorylated Thr68 of Chk2 mediate the MDC1-Chk2 interaction. Using MDC1 small interference RNA (siRNA) to downregulate MDC1, we found that MDC1 is important for chk2-dependent functions, such as intra-S phase checkpoint, p53 stabilization, and ionizing radiation-induced apoptosis. We also found that MDC1 regulates BRCA1 localization and phosphorylation following DNA damage. Downregulation of MDC1 resulted in defective G2/M checkpoint. These results suggest that MDC1 is an important mediator of DNA damage response and a potential tumor suppressor.

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Introduction

The current study investigates the functional interaction between Chk2 and Kiaa0170. Chk2 is a critical regulator of DNA damage response pathway and a tumor suppressor. Mutations in Chk2 confer an increased risk of breast cancer (1-3). However, the regulation of the Chk2-mediated pathway is still not clear. Our preliminary studies show that Kiaa0170 (renamed as MDC1, Mediator of DNA Damage Checkpoint Protein 1), a newly identified nuclear protein, associates with Chk2 after ionizing radiation (IR). We proposed to study the molecular mechanism of the MDC1-Chk2 interaction and the functional role of MDC1 in the Chk2-dependent DNA damage response pathway. These studies will provide new insight into the regulation of the DNA damage response pathway and the development of tumor. They might also reveal new strategies for tumor therapy and prevention.

Body

The specific aims 1is to define and characterize the interacting domains between Chk2 and MDC1. Previously, we have shown in the preliminary data that the GST-FHA domain of MDC1 efficiently pull-downed Chk2 in a DNA-damage dependent manner. To confirm that the MDC1 FHA domain interacts with Chk2 *in vivo*, we generated construct expressing S-tagged full-length MDC1 and MDC1 deleted of the FHA domain. As shown in Figure 1, deletion of the FHA domain abolished the Chk2-MDC1 interaction.

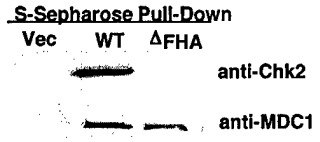

Figure 1. The FHA domain of MDC1 is required for the MDC1-Chk2 interaction *in vivo*. S-tagged wild-type MDC1 (WT) or MDC1 deleted of the FHA domain (ΔFHA) were expressed in HeLa cells. Following IR, Cell lysates were incubated with S- Sepharose and proteins retained were separated by SDS-PAGE and blotted with anti-Chk2 and anti-MDC1 antibodies.

These data suggest that the FHA domain of MDC1 is both sufficient and necessary for the MDC1-Chk2 interaction. FHA domains have been shown to bind phospho-Ser/Thr motif (4). Since MDC1 interacts Chk2 in a DNA-damage dependent manner, it is possible that MDC1 binds phosphorylated Chk2. The SQ/TQ rich region of Chk2 is hyper-phosphorylated following DNA damage. We generated cell lines stably express HA-tagged full-length Chk2 and Chk2 deleted of the SQ/TQ rich region. Using pull down assay with GST-MDC1 FHA domain, we found that the deletion of SQ/TQ rich region significantly decreased the MDC1-Chk2 interaction (See Appended \Publication 1, Nature Figure 2e). Importantly, Thr68 of Chk2, which is the major phosphorylation site of ATM following DNA damage, localized at the SQ/TQ rich region. We found that mutation of Thr68 to Ala abolished the MDC1-Chk2 interaction following IR (Figure 2).

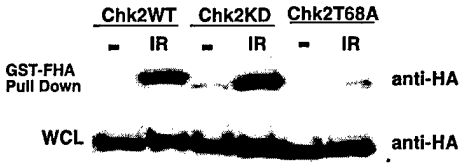

Figure 2. Phosphorylated Thr68 is required for the MDC1-Chk2 Interaction. HCT15 cells expressing HA-tagged wild-type Chk2 (Chk2WT), kinase-dead Chk2 (Chk2KD) or T68A Chk2 (Chk2T68A) were left untreated or treated with IR and lyzed 1hr later. Cell lysates were incubated with GST-MDC1 FHA domain immobilized on GSH-agarose, and protein retained on agarose were separated by SDS-PAGE and blotted with anti-HA antibodies. Whole cell lysates (WCL) were blotted to show the expression of Chk2 constructs.

These results suggest that MDC1 bind to phosphorylated Thr68 of Chk2. Further *in vitro* studies using Chk2 peptides confirmed that it is the FHA domain of MDC1 and the phosphorylated Thr68 mediate MDC1-Chk2 interaction (See Appendix 1, Nature Figure 2g-h). Therefore, both *in vitro* and in *vivo* studies defined the molecular mechanism of MDC1-Chk2 interaction proposed in Statement of Work Specific Aim 1.

We next investigated the functional role of MDC1 in Chk2-dependent DNA damage-signaling pathway, as proposed in Specific Aim 2. Here we used small inteference RNA (siRNA) to knockdown MDC1 and evaluated cellular response to DNA damage. Chk2 has been shown to regulate intra-S phase checkpoint, p53 stabilization and radiation induced checkpoint (5). Interestingly, we found that similar to Chk2-deficient cells, cells depleted of MDC1 show radioresistant DNA synthesis (RDS) phenotype, suggesting that MDC1 is involved in intra-S checkpoint (Figure3).

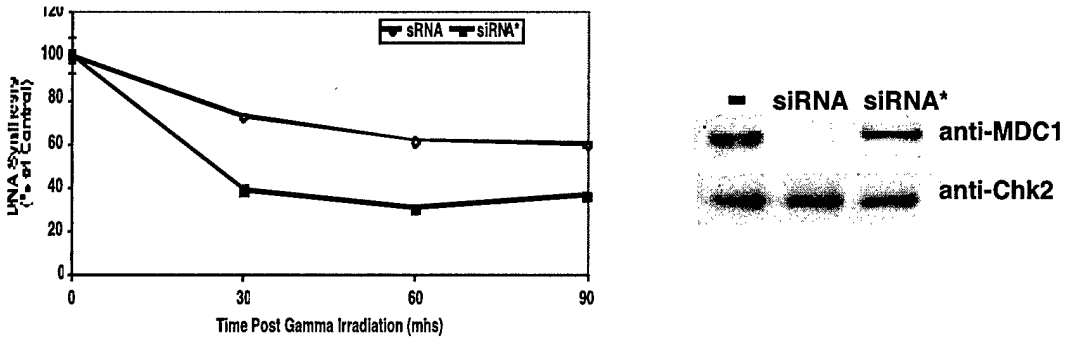

Figure3. A549 cells were transfected with control siRNA (siRNA*) or MDC1 siRNA (siRNA). 72 hrs later, cells were pulsed ^{3}H-thymidine and irradiated. Cells were harvested after indicated time, and DNA synthesis was evaluated (left panel). Western blot show the downregulation of MDC1 expression (right panel).

Furthermore, depletion of MDC1 resulted in defective p53 stabilization and IR-induced apoptosis (Figure 4).

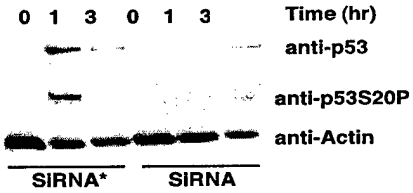

Figure 4. A549 cells were transfected with control siRNA (siRNA*) or MDC1 siRNA (siRNA). 72 hrs later, cells were irradiated and lyzed. Cell lysates were separated by SDS-PAGE, and blotted with anti-p53, anti-pS20p53 and anti-actin antibodies.

These results suggest that MDC1 regulated Chk2 dependent checkpoint activation and apoptosis. To further confirm that MDC1-Chk2 interaction is required for DNA damage response, we generated siRNA-resistant full length MDC1 (MDC1*) and MDC1 deleted of the FHA domain (MDC1dFHA*) by introducing silent mutations within the cDNA sequence that was targeted by siRNA. We show that reconstitute MDC1 knockdown cells with MDC1* restored IR-induced apoptosis, while reconstitution of MDC1dFHA* failed to do so (See Appended Publication 1, Nature Figure 4 b-c). These data established that the functional significance of the MDC1-Chk2 interaction as proposed in Statement of Work Specific Aim 2.

During the course of this investigation, we made additional findings. We found that MDC1 interacts with BRCA1, another critical suppressor of breast cancer (6). MDC1 interacts with BRCA1 constitutively, and colocalizes with BRCA1 at the sites of DNA damage (See Appended Publication 2, J. Biol. Chem., Figure 2A-2B). In addition, we found that MDC1 regulates BRCA1 foci formation and phosphoryaltion (Figure 5 and Appended Publication 2, J. Biol. Chem. Figure 3A).

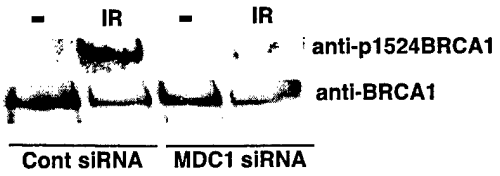

Figure 5. A549 cells were transfected with control siRNA or MDC1 siRNA. 72 hrs later, cells were irradiated and lyzed. BRCA1 was immunoprecipitated and blotted with anti-phosphoSer1524 BRCA1 and anti-BRCA1 antibodies.

In addition, depletion of MDC1 resulted in defective Chk1 activation and G2/M checkpoint (Figure 6, and Appended Publication 2, J. Biol. Chem. Figure 4B), similar to that of BRCA1-deficient cells.

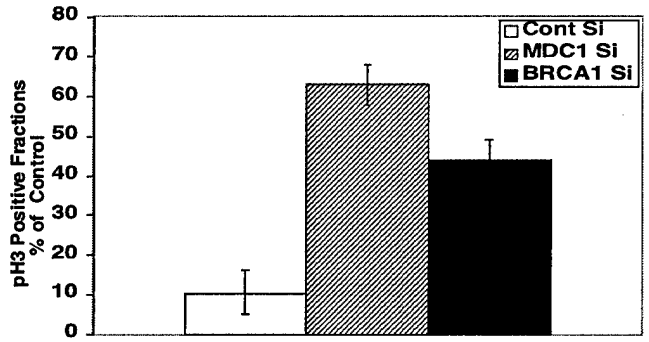

Figure 6. A549 cells were transfected with control siRNA, BRCA1 siRNA, or MDC1 siRNA (siRNA). 72 hrs later, cells were irradiated. Cells were harvested 1hr later and immunostained with anti-phosphoH3 antibodies and the percentage of mitotic cells were evaluated.

These data suggest that MDC1 is an upstream regulator of BRCA1 in DNA damage response pathway.

Key Research Accomplishments

MDC1 interacts with Chk2 in a DNA damage dependent manner.

MDC1 interacts with Chk2 through the FHA domain of MDC1 and phospho-Thr68 of Chk2.

MDC1 regulates Chk2-dependent functions: intra-S checkpoint, p53 stabilization and IR-induced apoptosis.

The Chk2-MDC1 interaction is important for Chk2 function.

MDC1 interacts with BRCA1 constitutively.

MDC1 regulates BRCA1 localization and phosphorylation following IR.

MDC1 regulates G2/M checkpoint and Chk1 activation following IR.

Reportable Outcomes

Publications:

1. Lou, Z., Minter-Dykhouse, K., Wu, X. and Chen, J. MDC1 Is Coupled to Activated Chk2 in Mammalian DNA Damage Response Pathways. ***Nature*** *421:957-961, 2003.*

2. Lou, Z., Chini, C., Minter-Dykhouse, K., and Chen, J. MDC1 Regulates BRCA1 Relocalization and Phosphorylation in DNA damage Checkpoint. ***J. Biol Chem.***, 278:13599-13603, 2003.

3. Lou, Z and Chen, J. BRCA proteins and DNA Damage Checkpoints. ***Frontiers in Bioscience.*** 8:S718-721, 2003.

4. Lou, Z and Chen, J. **Methods In Molecular Biology** (Human Press) : Use of siRNA to study the function of MDC1 in DNA damage responses. ***Methods in Molecular Biology, 281: 179-187.***

Presentation:

Lou, Z. MDC1, a mediator of DNA damage checkpoint control. 5th Annual Midwest DNA Repair Symposium. Rochester, MN, 2003

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Promoted to Instructor from Research Fellow

Conclusions

We characterized the interaction of MDC1 and Chk2, and found that the FHA domain of MDC1 and the phospho-Thr68 of Chk2 mediate the MDC1-Chk2 interaction. MDC1 also interacts with BRCA1 constitutively. We found that MDC1 regulates Chk2 and BRCA1-dependent functions, such as intra-S checkpoint, G2/M checkpoint and IR-induced apoptosis. Importantly, other groups have identified MDC1 as a critical player of DNA damage response (7-12). These studies confirmed our findings and established MDC1 as an important mediator of DNA damage response. Given the important role of BRCA1 and Chk2 in tumor suppression, it is very possible that MDC1 acts as a tumor suppressor. Although not stated in the approved Statement of Work, we are generating MDC1 knockout mice as animal model to study the tumor suppressor function of MDC1. We will continue work on Specific Aim 3: to screen human tumor cell line for MDC1 mutations. In summery, our studies revealed novel mechanisms of the DNA damage response pathway and will provide new insight into the maintenance of genomic stability. Animal models with knockout mice will elucidate how dysfunction of the Chk2-MDC1 pathway contributes to the development of tumor.

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MDC1 is coupled to activated CHK2 in mammalian DNA damage response pathways

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Forkhead-homology-associated (FHA) domains function as protein–protein modules that recognize phosphorylated serine/threonine motifs[1–5]. Interactions between FHA domains and phosphorylated proteins are thought to have essential roles in the transduction of DNA damage signals; however, it is unclear how FHA-domain-containing proteins participate in mammalian DNA damage responses. Here we report that a FHA-domain-containing protein—mediator of DNA damage checkpoint protein 1 (MDC1; previously known as KIAA0170)—is involved in DNA damage responses. MDC1 localizes to sites of DNA breaks and associates with CHK2 after DNA damage. This association is mediated by the MDC1 FHA domain and the phosphorylated Thr 68 of CHK2. Furthermore, MDC1 is phosphorylated in an ATM/CHK2-dependent manner after DNA damage, suggesting that MDC1 may function in the ATM–CHK2 pathway. Consistent with this hypothesis, suppression of MDC1 expression results in defective S-phase checkpoint and reduced apoptosis in response to DNA damage, which can be restored by the expression of wild-type MDC1 but not MDC1 with a deleted FHA domain. Suppression of MDC1 expression results in decreased p53 stabilization in response to DNA damage. These results suggest that MDC1 is recruited through its FHA domain to the activated CHK2, and has a critical role in CHK2-mediated DNA damage responses.

The DNA-damage-induced cell-cycle checkpoints and apoptosis are critical for the maintenance of genomic stability[6,7]. Many proteins involved in DNA damage responses contain functional domains such as FHA and BRCA1 carboxy-terminal (BRCT) domains[1,8]. FHA and BRCT domains are molecular modules involved in protein–protein interaction. FHA domains specifically recognize motifs that contain phosphorylated serine/threonine in a manner similar to the SH2-phosphotyrosine interaction[2–5]. A well-studied example of the FHA-domain-mediated interaction is the Rad9–Rad53 interaction in *Saccharomyces cerevisiae*[9–11], which is critical for the activation of DNA damage pathways in yeast. However, except for the homo-oligomerization of checkpoint kinase 2 (CHK2)[12,13], such FHA-domain-mediated interactions are not documented in mammalian cells. More importantly, the role of FHA domains in DNA damage signal transduction remains to be established in mammals.

MDC1 is a protein that contains both FHA and BRCT domains. Because the BRCT domain of MDC1 shares close sequence homology with that of BRCA1 and yeast Rad9, we examined whether MDC1 is involved in DNA damage response pathways in mammalian cells. Many proteins involved in DNA damage response pathways form nuclear foci after DNA damage (for example, BRCA1 and CHK2)[14,15]. Therefore, we first investigated the nuclear localization of MDC1 before and after DNA damage. MDC1 normally localized in the nuclei without any apparent structures. However, after γ-irradiation, MDC1 relocalized to nuclear foci (Fig. 1a) and localized together with phosphorylated H2AX (γH2AX) foci, suggesting that MDC1 relocalizes to the sites of DNA damage[16,17]. In addition, similar to many other signalling proteins in DNA damage response pathways, MDC1 migrates slower after γ-irradiation (Fig. 1b). Treatment of MDC1 immunoprecipitates with λ-phosphatase reverses this mobility shift (Fig. 1b), suggesting that MDC1 is phosphorylated after γ-irradiation. To determine whether the phosphorylation of MDC1 depends on ataxia–telangiectasia mutated (ATM), K562 cells and ATM-deficient GM03189D cells were either irradiated or left untreated. In contrast to that in K562 cells, no mobility shift of MDC1 was observed in GM03189D cells after γ-irradiation (Fig. 1c), suggesting that the phosphorylation of MDC1 is ATM-dependent.

To explore the role of MDC1 in DNA damage pathways, we examined whether MDC1 would associate with proteins involved in DNA damage response pathways. As shown in Fig. 2a, MDC1 associated with CHK2 only after DNA damage. This interaction is not interrupted by ethidium bromide (data not shown), suggesting that the MDC1–CHK2 interaction is not mediated by DNA. Furthermore, MDC1 localized together with the phosphorylated Thr 68 residue of CHK2 after DNA damage (Fig. 2b). As MDC1 interacts with the active form of CHK2 (Thr-68-phosphorylated CHK2) after DNA damage, we investigated whether the phosphorylation of MDC1 requires CHK2. CHK2-deficient HCT15 cells and HCT15 cells stably transfected with either wild-type CHK2 or kinase-dead CHK2 were irradiated or left untreated. No mobility shift of MDC1 was observed in HCT15 cells after γ-irradiation. However, mobility shift of MDC1 was restored in HCT15 cells reconstituted with wild-type CHK2, but not in cells reconstituted with kinase-dead CHK2 (Fig. 2c).

To explore further the interaction between MDC1 and CHK2, we generated glutathione *S*-transferase (GST) fusion proteins that contained either the MDC1 FHA domain or the MDC1 BRCT domain, as FHA and BRCT domains are involved in protein–protein interactions. Figure 2d shows that GST–MDC1(FHA), but not GST–MDC1(BRCT), interacted with CHK2 after DNA damage. The *in vitro* interactions of GST–MDC1(FHA) and CHK2 are

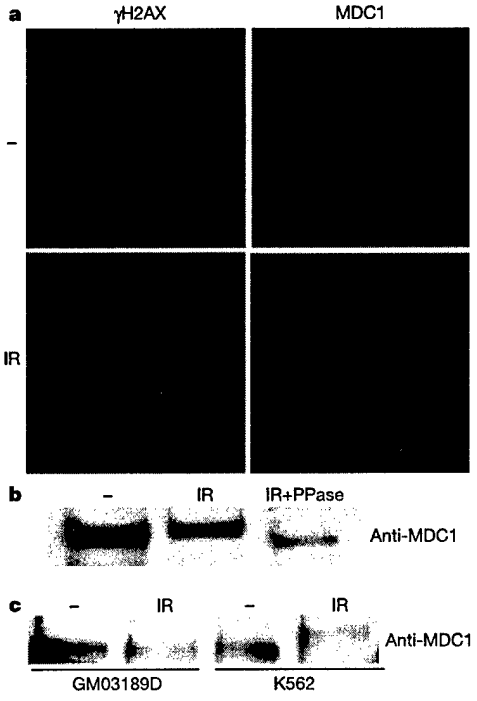

Figure 1 MDC1is phosphorylated and relocalizes to nuclear foci after DNA damage. **a**, 293T cells were left untreated (top panels) or treated with 10 Gy γ-irradiation (IR; bottom panels), and 10 h later they were stained with anti-γH2AX antibodies (left) or anti-MDC1 antibodies (right). **b**, MDC1 was immunoprecipitated from untreated or γ-irradiated (30 Gy) K562 cells and incubated with buffer or λ-phosphatase (PPase), then blotted with anti-MDC1 antibodies. **c**, K562 cells and GM03189D cells (ATM deficient) were γ-irradiated (30 Gy) or left untreated, then blotted with anti-MDC1 antibodies.

specific and depend on the integrity of the FHA domain of MDC1, as mutations in highly conserved residues within this domain (R58A, S72A, N96G97T98/AAA) abolished or reduced the interaction between MDC1 and CHK2 (data not shown). To test whether the MDC1 FHA domain is required for the MDC1–CHK2 interaction *in vivo*, we generated constructs encoding S-tagged wild-type MDC1 or MDC1 with a deleted FHA domain (MDC1ΔFHA). 293T cells transfected with these constructs were irradiated and lysates were incubated with S-Sepharose to pull-down wild-type or the deletion mutant of MDC1. As shown in Fig. 2e, in contrast with wild-type MDC1, MDC1ΔFHA failed to interact with CHK2. These results suggest that the damage-dependent interaction between MDC1 and CHK2 depends on the FHA domain of MDC1.

To examine which region of CHK2 is important for the MDC1–CHK2 interaction, constructs encoding haemagglutinin (HA)-tagged wild-type CHK2 (CHK2WT) and CHK2 deleted of the S/TQ-rich region (CHK2ΔS/TQ) were stably transfected into CHK2-deficient HCT15 cells. Deletion of the S/TQ-rich region of CHK2 completely abolished the MDC1–CHK2 association, suggesting that the S/TQ-rich region of CHK2 is required for MDC1 binding (data not shown). The S/TQ-rich region of CHK2 contains multiple potential phosphorylation sites for ATM/ataxia–telangiectasia and Rad3 related (ATR) kinases. Among them, Thr 68 has been shown to be an ATM phosphorylation site that is critical for CHK2 activation[18–22]. Notably, as determined by degenerate peptide library studies, the CHK2 sequence surrounding phosphorylated Thr 68 is a predicated recognition sequence of the MDC1 FHA domain[4]. Therefore, we next tested whether phosphorylated Thr 68 of CHK2 is the site recognized by the MDC1 FHA domain. Again, *in vitro* pull-down assay using GST–MDC1(FHA) was performed using HCT15 cells stably transfected with CHK2WT, kinase-dead CHK2 (CHK2KD) or CHK2 containing a T68A mutation (CHK2T68A). Whereas the kinase-dead mutant of CHK2 still interacted with MDC1 in a DNA-damage-dependent manner (Fig. 2f), the T68A mutation abolished the MDC1–CHK2 interaction. These results suggest that the phosphorylation of CHK2 at Thr 68 by ATM, but not CHK2 autophosphorylation sites, is critical for MDC1 binding.

To confirm further the critical role of Thr-68-phosphorylated CHK2 in the MDC1–CHK2 interaction, synthetic CHK2 peptides representing CHK2 amino acid sequence surrounding Thr 68 (residues 64–73) were prepared with Thr 68 as either a phosphorylated (p-T68) or non-phosphorylated (T68) residue. If phosphorylated Thr 68 and the surrounding sequence of CHK2 are truly recognized by the MDC1 FHA domain, there would be competition between the p-T68 peptide and endogenous CHK2 for binding to GST–MDC1(FHA). As expected, p-T68 peptide, but not T68 peptide, effectively blocked the binding of endogenous CHK2 to GST–MDC1(FHA) after DNA damage (Fig. 2g). As a negative control, a phosphorylated peptide containing CHK2 Ser 516 (an autophosphorylation site; our own unpublished observation) did not block the binding of CHK2 with MDC1 (Fig. 2g). Furthermore, when p-T68 or T68 peptides were conjugated to Sepharose and incubated with cell lysates, only p-T68 peptide was able to interact with MDC1 (Fig. 2h). To demonstrate the direct interaction between the MDC1 FHA domain and the phosphorylated Thr 68 site of CHK2, T68 or p-T68 peptide conjugated with Sepharose were

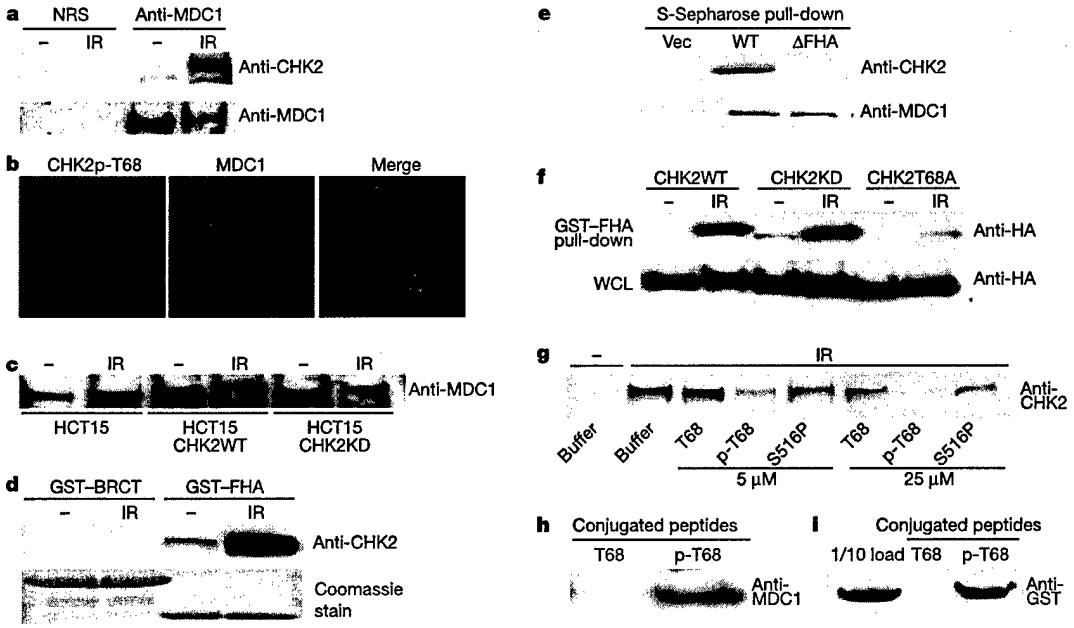

Figure 2 MDC1 interacts with CHK2 through the MDC1 FHA domain and the phosphorylated Thr 68 of CHK2. **a**, Cell lysates from K562 cells untreated or treated with 30 Gy γ-irradiation were subjected to immunoprecipitation with normal rabbit serum (NRS) or anti-MDC1 antibodies, and the immunoprecipitates were analysed with anti-CHK2 antibody. **b**, 293T cells were irradiated (10 Gy) and 10 h later stained with the indicated antibodies. **c**, HCT15 cells (CHK2 deficient) and HCT15 cells stably transfected with wild-type CHK2 or kinase-dead CHK2 were γ-irradiated (30 Gy) or left untreated, and cell lysates were then blotted with anti-MDC1 antibodies. **d**, *In vitro* binding assay. Beads bound with GST–MDC1(FHA) and GST–MDC1(BRCT) were incubated with lysates from control or γ-irradiated (30 Gy) K562 cells. Binding of CHK2 was analysed by immunoblotting. **e**, 293T cells were transfected with the indicated constructs and treated with 30 Gy γ-irradiation. The association of S-tagged MDC1 with CHK2 was assessed using the indicated antibodies. WT, wild type. **f**, *In vitro* binding assays. HCT15 cells stably transfected with the indicated HA-tagged constructs were treated with 30 Gy γ-irradiation or left untreated. Beads bound with GST–MDC1(FHA) were used to pull-down HA-tagged wild-type or mutants, and were blotted with anti-HA antibodies. Whole cell lysates (WCL) were blotted with anti-HA antibodies (bottom panel). **g**, Peptide competition assay. K562 cell lysates were incubated with beads bound with GST–MDC1(FHA) plus the indicated concentration of CHK2 peptides. The remaining CHK2 on the beads was detected by anti-CHK2 antibodies. **h**, **i**, Non-phosphorylated or phosphorylated Thr peptides (T68 and or p-T68, respectively) conjugated to Sepharose beads were incubated with the lysate prepared from untreated K562 cells (**h**), or purified GST–MDC1(FHA) (**i**). Proteins bound on beads were analysed with anti-MDC1 antibodies (**h**) or anti-GST antibodies (**i**).

incubated with purified GST–MDC1(FHA). As shown in Fig. 2i, only p-T68 peptide strongly binds to the MDC1 FHA domain. These data suggest that phosphorylated Thr 68 of CHK2 acts as a docking site for MDC1.

The interaction of MDC1 and CHK2 after γ-irradiation suggests that MDC1 may be involved in CHK2-mediated DNA damage responses. To investigate whether MDC1 functions in the ATM–CHK2 pathway, we used the small interfering RNA (siRNA) technique to suppress MDC1 expression[23]. Transfection of MDC1 siRNA resulted in the suppression of MDC1 in A549 cells (Fig. 3a, b).

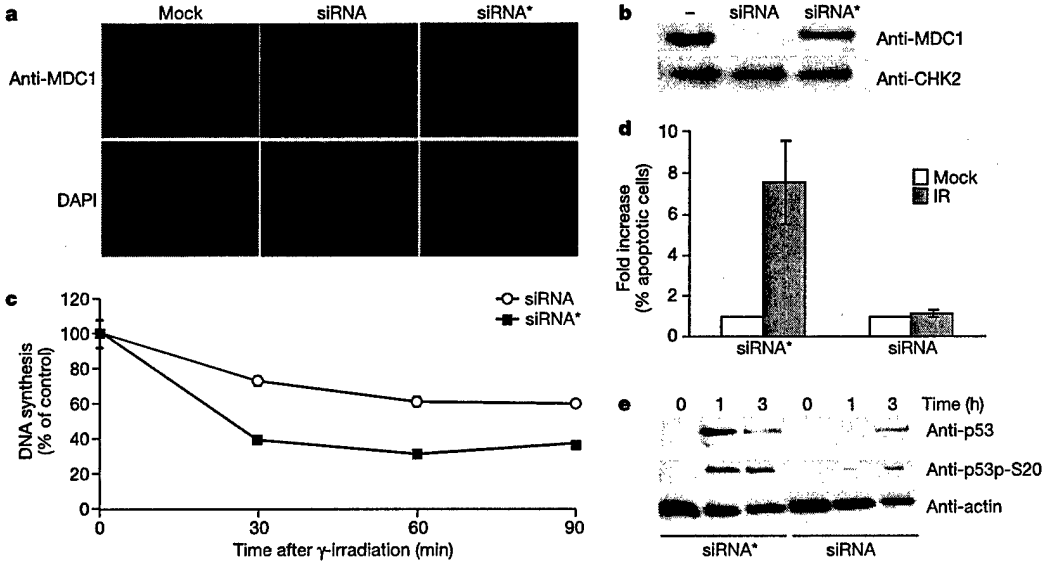

Figure 3 MDC1 is required for CHK2-mediated DNA damage responses. **a**, **b**, A549 cells were mock-transfected or transfected with MDC1 siRNA or control siRNA (siRNA*) twice. Seventy-two hours after the initial transfection, cells were immunostained with anti-MDC1 antibodies (**a**) or blotted with anti-MDC1 antibodies or CHK2 antibodies as a control (**b**). DAPI, 4,6-diamidino-2-phenylindole. **c**, A549 cells transfected with MDC1 siRNA or control siRNA as described for **a** and **b**. Cells were γ-irradiated (20 Gy) or left untreated, and DNA synthesis was assessed. **d**, A549 cells were transfected with either control siRNA or MDC1 siRNA, then left untreated or irradiated (5 Gy). Thirty-six hours later, cells were collected and evaluated for apoptosis. **e**, A549 cells were transfected with either control siRNA or MDC1 siRNA, then irradiated (5 Gy). Cells were lysed at the time indicated and blotted for p53, Ser-20-phosphorylated (p-S20) p53 or actin.

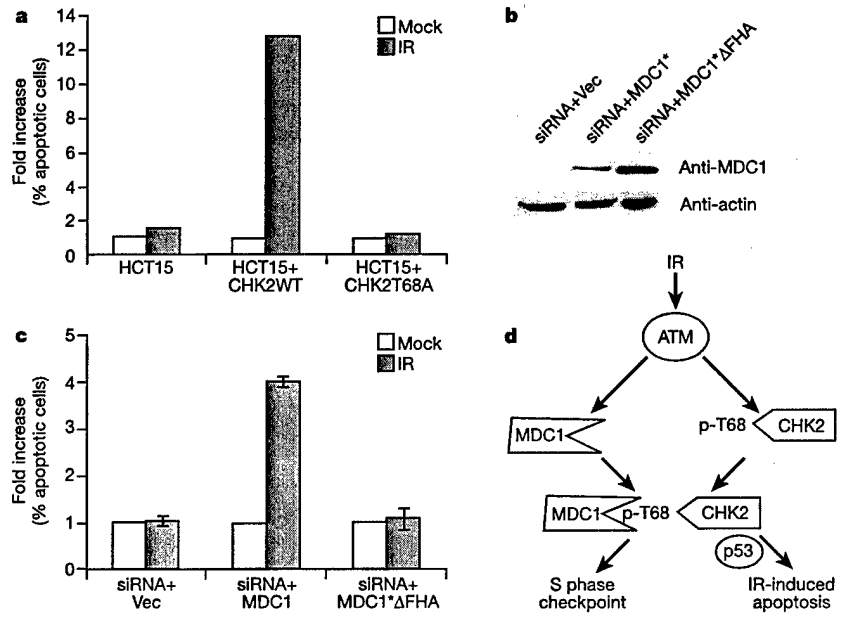

Figure 4 The functional interaction of CHK2 and MDC1 is required for CHK2-mediated DNA damage responses. **a**, HCT15 cells (CHK2 deficient) and HCT15 cells stably transfected with wild-type CHK2 or CHK2T68A were γ-irradiated (10 Gy) or left untreated. Thirty-six hours later, cells were collected and evaluated for apoptosis. **b**, **c**, A549 cells were transfected with MDC1 siRNA, and 24 h later they were transfected with constructs encoding green fluorescent protein (GFP) and either pCDNA3 vector, pCDNA3 encoding siRNA-resistant wild-type MDC1 (MDC1*) or MDC1ΔFHA (MDC1*ΔFHA). Thirty-six hours after the initial transfection, cells were collected and blotted for MDC1 expression (**b**), or sorted for GFP-positive cells (**c**). The GFP-positive cells were then either irradiated (5 Gy) or left untreated, and 48 h later, cells were evaluated for apoptosis. **d**, Model of how MDC1 interacts with activated CHK2 and functions in CHK2-mediated DNA damage responses.

Similar effects were observed in Hela cells, BJ cells and 293 T cells (data not shown). We also prepared control siRNA by introducing four point mutations into MDC1 siRNA (siRNA*). Transfection of control siRNA did not affect the level of MDC1, indicating the specificity of MDC1 siRNA (Fig. 3a, b). To examine whether suppression of MDC1 expression affects CHK2-mediated DNA damage responses, we examined downstream events regulated by CHK2. One such event is the ATM/CHK2-dependent temporal inhibition of DNA synthesis in S phase[24,25]. In ATM-deficient and CHK2-deficient cells, radioresistant DNA synthesis (RDS) occurs owing to the defective S-phase checkpoint[24,25]. We examined DNA synthesis after γ-irradiation in cells transfected with MDC1 siRNA or control siRNA. There was a 60–70% inhibition of DNA synthesis in cells transfected with control siRNA after γ-irradiation (Fig. 3c). However, in cells transfected with MDC1 siRNA, there was only 30–40% inhibition, consistent with an RDS phenotype (Fig. 3c). Similar RDS phenotype was also observed in BJ cells transfected with MDC1 siRNA (data not shown). To evaluate further the role of MDC1 in CHK2-mediated DNA responses, we evaluated the radiation-induced apoptosis. Recent studies suggest that the radiation-induced apoptosis requires CHK2 activity[26–28]. As shown in Fig. 3d, γ-irradiation induced apoptosis in A549 cells transfected with control siRNA. This γ-irradiation-induced apoptosis was abolished when A549 cells were transfected with MDC1 siRNA. These results indicate that MDC1 has a critical involvement in CHK2-mediated DNA damage responses. To explore further downstream molecules regulated by MDC1, we examined p53 stabilization after γ-irradiation, as CHK2 regulates radiation-induced apoptosis by phosphorylating p53 at Ser 20, which leads to the stabilizaton of p53 (refs 26–30). Compared with cells transfected with control siRNA, cells transfected with MDC1 siRNA show decreased and delayed p53 stabilization after γ-irradiation (Fig. 3e). Consistent with p53 stabilization, phosphorylation of p53 at Ser 20 is also decreased and delayed in cells transfected with MDC1 siRNA (Fig. 3e). These results indicate that MDC1 was able to regulate γ-irradiation-induced apoptosis by influencing p53 stabilization.

To establish specifically that the CHK2–MDC1 interaction is required for the DNA damage responses shown above, we evaluated γ-irradiation-induced apoptosis in HCT15 cells stably transfected with wild-type CHK2 or CHK2 with a mutation at Thr 68 (T68A), which could not associate with MDC1. As shown in Fig. 4a, HCT15 cells show defective apoptosis in response to γ-irradiation, which was restored by the reconstitution of wild-type CHK2, but not CHK2T68A. Furthermore, we generated MDC1 constructs (full-length and ΔFHA) that are resistant to siRNA targeting by introducing silent mutations at the region targeted by siRNA. Co-transfection of these constructs with MDC1 siRNA can restore the expression of MDC1 (Fig. 4b). Co-transfection of MDC1 siRNA and siRNA-resistant full-length MDC1 (MDC1*), but not siRNA-resistant MDC1ΔFHA (MDC1*ΔFHA), restored γ-irradiation-induced apoptosis (Fig. 4c). These results suggest that the interaction of CHK2 and MDC1 is critical for CHK2-mediated DNA damage responses.

We have shown that MDC1 is recruited to activated CHK2 in response to DNA damage. This recruitment is conducted through the interaction between the FHA domain of MDC1 and the phosphorylated Thr 68 site of CHK2. MDC1 is phosphorylated in an ATM- and CHK2-dependent manner and is involved in S-phase checkpoint and radiation-induced apoptosis (Fig. 4d). These results indicate that MDC1 has an important role in CHK2-mediated DNA damage responses. Furthermore, these data demonstrate the importance of the FHA domain–phosphoprotein interaction in the mammalian DNA damage signalling pathways. □

Methods

Plasmids and siRNAs

MDC1 complementary DNA was provided by T. Nagase (Kazusa DNA Research Institute). Wild-type MDC1 or MDC1ΔFHA was cloned into a pIRES2 vector containing S-protein tag. siRNA-resistant constructs were made by introducing a silent mutation at the MDC1 coding region (70–75; TTGAGG to CTTAGA). The MDC1 FHA and BRCT domains were cloned into the pGEX5.3 vector for bacterial expression of GST fusion proteins. CHK2 constructs have been described previously[15]. MDC1 siRNAs were synthesized by Xerogon Inc. The siRNA duplexes were 21 base pairs including a 2-deoxnucleotide overhang. The coding strand of MDC1 siRNA (MDC1 cDNA 58–76) was UCCAGUGAAUCCUUGAGGUdTdT; the control siRNA was UUCAAUAAAUUCUUGAGGUdTdT.

Antibodies and cell lines

MDC1 antibodies were raised against GST fusion proteins containing amino-terminal residues 1–150 or 151–484 of MDC1. CHK2 antibodies have been previously described[15]. Anti-γH2AX antibodies were generated as previously described[15]. Antibodies against p53 and phosphorylated Ser 20 of p53 were purchased from Cell Signaling.

All cells were obtained from American Tissue Culture Collections and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. HCT15 stable cell lines expressing wild-type or mutant CHK2 were generated by neomycin selection.

Immunoprecipitation, immunoblotting and immunostaining

We performed cell lysate preparation, immunoprecipitation, immunoblotting and immunostaining as described[14].

In vitro pull-down assay and peptide competition assay

GST fusion proteins were bound to glutathione-Sepharose overnight at 4 °C. The beads were washed with PBS twice and incubated with cell lysates for 1 h at 4 °C. For peptide competition assay, the indicated concentration of T68 peptide (CETVSTQELYS) and p-T68 peptide (CETVS-pT-QELYS) was added to cell lysates before incubation with beads bound to GST fusion proteins.

siRNA transfection, RDS assay and apoptosis assay

SiRNA transfection was performed as described previously[23]. Seventy-two hours after initial transfection, cells were γ-irradiated (20 Gy) or left untreated. Cells were then pulsed with [^{3}H]thymidine after the indicated times, and collected 30 min later. For apoptosis assay, cells were irradiated at the indicated doses, and 36 h later fixed and stained with Hoechst. Cells were then scored for apoptosis.

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MDC1 is a mediator of the mammalian DNA damage checkpoint

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To counteract the continuous exposure of cells to agents that damage DNA, cells have evolved complex regulatory networks called checkpoints to sense DNA damage and coordinate DNA replication, cell-cycle arrest and DNA repair[1]. It has recently been shown that the histone H2A variant H2AX specifically controls the recruitment of DNA repair proteins to the sites of DNA damage[2–4]. Here we identify a novel BRCA1 carboxy-terminal (BRCT) and forkhead-associated (FHA) domain-containing protein, MDC1 (mediator of DNA damage checkpoint protein 1), which works with H2AX to promote recruitment of repair proteins to the sites of DNA breaks and which, in addition, controls damage-induced cell-cycle arrest checkpoints. MDC1 forms foci that co-localize extensively with γ-H2AX foci within minutes after exposure to ionizing radiation. H2AX is required for MDC1 foci formation, and MDC1 forms complexes with phosphorylated H2AX. Furthermore, this interaction is phosphorylation dependent as peptides containing the phosphorylated site on H2AX bind MDC1 in a phosphorylation-dependent manner. We have shown by using small interfering RNA (siRNA) that cells lacking MDC1 are sensitive to ionizing radiation, and that MDC1 controls the formation of damage-induced 53BP1, BRCA1 and MRN foci, in part by promoting efficient H2AX phosphorylation. In addition, cells lacking MDC1 also fail to activate the intra-S phase and G2/M phase cell-cycle checkpoints properly after exposure to ionizing radiation, which was associated with an inability to regulate Chk1 properly. These results highlight a crucial role for MDC1 in mediating transduction of the DNA damage signal.

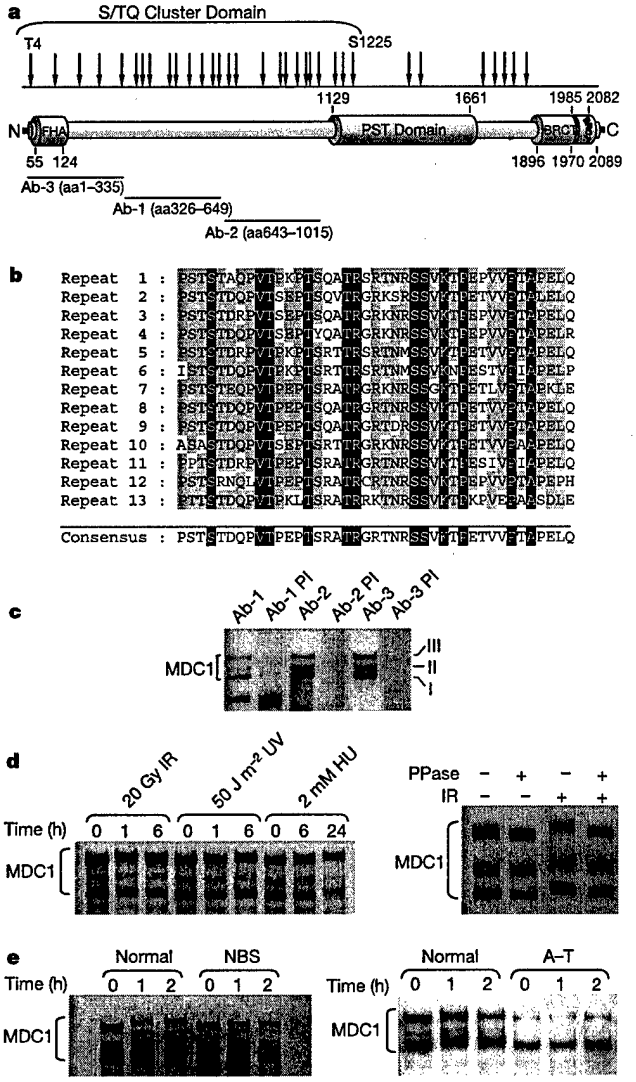

Figure 1 MDC1 is phosphorylated in response to DNA damage and DNA replication stress. **a**, A diagrammatic representation of the MDC1 protein. The amino acids encompassing each domain are indicated. PST indicates proline/serine/threonine repeat domain. The red arrows indicate potential phosphatidylinositol-3-OH kinase-like kinase phosphorylation motifs (SQ/TQ). The fragments of the MDC1 protein used to make anti-MDC1 antibodies (Ab-1, Ab-2, Ab-3) are indicated. **b**, Alignment of the 41-amino-acid repeat sequence that composes the PST domain. Black shaded boxes indicate conserved amino acids and grey boxes indicate similar amino acids. **c**, Recognition of three isoforms (I, II and III) of human MDC1. PI refers to pre-immune serum. **d**, DNA damage-induced phosphorylation of MDC1. Cells were treated with 20 Gy IR, 50 J m^{-2} UV or 2 mM HU and harvested at the times indicated. IR-treated cell extracts were also incubated with and without λ protein phosphatase (λ PPase). **e**, MDC1 is phosphorylated in response to IR in an ATM- and Nbs1-dependent manner. Normal, NBS and A–T lymphoblasts were irradiated with 20 Gy of IR and harvested at the times indicated.

Mediators are an emerging class of checkpoint proteins involved in transducing the DNA damage signal. The prototypical mediator

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Mediator of DNA Damage Checkpoint Protein 1 Regulates BRCA1 Localization and Phosphorylation in DNA Damage Checkpoint Control*

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BRCA1 is a tumor suppressor involved in DNA repair and damage-induced checkpoint controls. In response to DNA damage, BRCA1 relocalizes to nuclear foci at the sites of DNA lesions. However, little is known about the regulation of BRCA1 relocalization following DNA damage. Here we show that mediator of DNA damage checkpoint protein 1 (MDC1), previously named NFBD1 or Kiaa0170, is a proximate mediator of DNA damage responses that regulates BRCA1 function. MDC1 regulates ataxia-telangiectasia-mutated (ATM)-dependent phosphorylation events at the site of DNA damage. Importantly down-regulation of MDC1 abolishes the relocalization and hyperphosphorylation of BRCA1 following DNA damage, which coincides with defective G_2/M checkpoint control in response to DNA damage. Taken together these data suggest that MDC1 regulates BRCA1 function in DNA damage checkpoint control.

FHA[1] and BRCT domains are functional modules that are involved in protein-protein interaction (1–3). Many proteins involved in the DNA damage response pathway, such as mammalian BRCA1, 53BP1, Chk2, NBS1, yeast Rad9, and Rad53, contain FHA or BRCT domains. Furthermore mutations within FHA and BRCT domains have been associated with tumorigenesis (4).[2] These findings suggest important roles of FHA and BRCT domains in DNA damage response pathways.

Kiaa0170 or NFBD1 (nuclear factor with BRCT domains protein 1) is a nuclear protein that contains both FHA and BRCT domains. Our initial studies on Kiaa0170 and studies from other laboratories have shown that Kiaa0170 forms nuclear foci at the sites of DNA damage and is phosphorylated in an ATM-dependent manner (5–7). Furthermore Kiaa0170 functions as a critical regulator in DNA damage signaling pathways (7, 8).[3] Therefore, Kiaa0170 has been renamed as mediator of DNA damage checkpoint protein 1 (MDC1) to better reflect its role in DNA damage checkpoint controls.[4] In this study, we further explored the role of MDC1 in ATM-dependent DNA damage response pathways.

EXPERIMENTAL PROCEDURES

Plasmids and Small Interfering RNAs (siRNAs)—MDC1 cDNA was kindly provided by Dr. T. Nagase from Kazusa DNA Research Institute (Chiba, Japan). MDC1 siRNAs were synthesized by Xerogon Inc. (Huntsville, AL). The siRNA duplexes were 21 base pairs including a two-deoxynucleotide overhang. The coding strand of MDC1 siRNA1 was UCCAGUGAAUCCUUGAGGU*dTdT*, and the coding strand of MDC1 siRNA2 was ACAACAUGCAGAGAUUGAA*dTdT*. The coding strand of BRCA1 siRNA was GGAACCUGUCTCCACAAAG*dTdT*, and the control siRNA was UUCAAUAAAUUCUUGAGGU*dTdT*.

Antibodies and Cell Lines—MDC1 antibodies were raised against glutathione *S*-transferase fusion proteins containing N-terminal residues 1–150 or 151–484 of MDC1. Anti-phospho-H2AX (γH2AX) and anti-BRCA1 antibodies were generated as described previously (8). Anti-p1524BRCA1 antibodies were kindly provided by Dr. KumKum Khanna. Anti-pS317Chk1 antibodies and anti-pATM/ATR were purchased from Cell Signaling. Anti-Chk1 mAb was purchased from Santa Cruz Biotechnology. Anti-phospho-histone 3 antibodies were purchased from Upstate Biotechnology. All cells were obtained from American Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Immunoprecipitation, Immunoblotting, and Immunostaining—Cell lysate preparation, immunoprecipitation, immunoblotting, and immunostaining were performed as described before (8).

siRNA Transfection—siRNA transfection was performed as described previously (9). Briefly, cells were grown in six-well plate to 30% confluence and immediately before transfection washed with serum-free medium, and 800 μl of serum-free medium were added per well. For each well, 200 nM siRNA was mixed with 5 μl of Oligofectamine (Invitrogen) in 200 μl of serum-free medium. The mixtures were incubated for 20 min at room temperature and then added to cells. Serum was added 4 h later to a final concentration of 10%. 24 h after the initial transfection, a second transfection was performed in the same way as the previous one. 72 h after initial transfection, cells were treated and harvested as indicated.

Phospho-H3 Staining—siRNA-transfected cells were γ irradiated (2 Gy) or left untreated. Cells were then stained with anti-phospho-H3 antibodies, and phospho-H3-positive cells were evaluated by counting or fluorescence-activated cell sorter.

RESULTS AND DISCUSSION

To test the role of MDC1 in ATM-dependent phosphorylation events, we used siRNA technology (9) to down-regulate MDC1 (Fig. 1*A*) and monitored ATM-dependent phosphorylation events by immunofluorescence staining (Fig. 1*B*). An antibody raised against phosphoepitope substrates of ATM or ATR has been shown to specifically recognize ATM/ATR-dependent phosphorylation events (10). As shown in Fig. 1*B*, in cells transfected with control siRNA, ionizing radiation (IR)-induced nuclear foci were present in most irradiated cells, suggesting the accumulation of phosphorylated ATM substrates at the

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[1] The abbreviations used are: FHA, forkhead-associated; BRCT, BRCA1 C-terminal; MDC1, mediator of DNA damage checkpoint protein 1; ATM, ataxia-telangiectasia-mutated; ATR, ATM and Rad3-related; siRNA, small interfering RNA; H3, histone 3; Gy, gray; IR, ionizing radiation; γH2AX, phospho-H2AX; BARD1, BRCA1-associated RING domain.

[2] Breast Cancer Information Core at research.nhgri.nih.gov/bic/ on the World Wide Web.

[3] S. Jackson and S. Elledge, personal communication.

[4] S. J. Elledge and S. P. Jackson, personal communication.

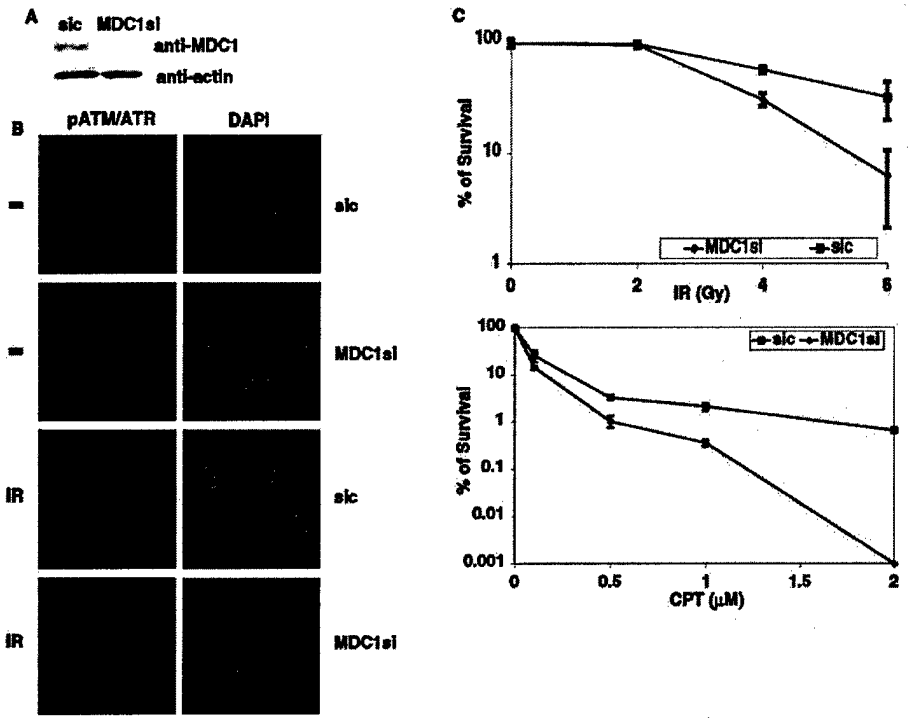

FIG. 1. **MDC1 is involved in ATM-dependent phosphorylation events.** *A* and *B*, HeLa cells were transfected with control siRNA (*sic*) or MDC1 siRNA. After 72 h, cells were lysed and blotted with anti-MDC1 (*upper panel*) or anti-actin antibodies (*lower panel*) (*A*), irradiated (10 Gy) or left untreated, and 16 h later stained with anti-phospho-ATM/ATR substrate antibodies and 4,6-diamidino-2-phenylindole (*DAPI*) (*B*). *C*, HeLa cells were transfected with control siRNA (*sic*) or MDC1 siRNA and treated with IR or camptothecin (*CPT*). Colony formation was determined 2 weeks later. *MDC1si*, MDC1 siRNA.

sites of DNA breaks. However, down-regulation of MDC1 abolished these IR-induced foci. These results suggest that MDC1 regulates ATM/ATR-dependent phosphorylation events upon DNA damage. Furthermore down-regulation of MDC1 resulted in increased sensitivity to IR and camptothecin (Fig. 1*C*), implying that MDC1 is required for cell survival following DNA double-stranded breaks. Together these data suggest that MDC1 is involved in ATM-dependent DNA damage response pathways.

In agreement with the role of MDC1 in ATM/ATR-dependent pathways, down-regulation of MDC1 also partially decreased γH2AX foci staining after IR.[5] This is different from a previous report (11) that showed intact γH2AX foci formation in cells transfected with MDC1 siRNA. This discrepancy could be due to the extent of MDC1 down-regulation by different siRNAs used. On the other hand, consistent with the previous report (11), we also observed that down-regulation of H2AX abolished MDC1 foci formation in response to DNA damage.[5] These findings suggest that H2AX phosphorylation and MDC1 foci formation depend on each other, possibly by forming a positive feedback loop: γH2AX recruits MDC1 to the sites of DNA damage, which in turn enhances the phosphorylation of H2AX by ATM.

These findings also raise the possibility that γH2AX and MDC1 cooperate as early signaling molecules to amplify ATM-dependent DNA damage signals. In response to DNA damage, BRCA1 (12) and many other proteins involved in DNA damage responses, such as MRE11/NBS1/Rad50 (13), 53BP1 (8, 14, 15), and Chk2 (16), form nuclear foci. These nuclear foci colocalize with γH2AX foci, which reside at the sites of DNA damage (17, 18). MDC1 also redistributes to nuclear foci in response to DNA damage (5–7, 11). Similarly MDC1 foci colocalized with BRCA1 foci (Fig. 2*A*). Furthermore we found that MDC1 co-immunoprecipitated with BRCA1. This interaction is constitutive, occurring independently of DNA damage (Fig. 2*B*). Given that BRCA1 forms a stable heterodimeric complex with BARD1 *in*

[5] Z. Lou, C. C. S. Chini, K. Minter-Dykhouse, and J. Chen, unpublished observation.

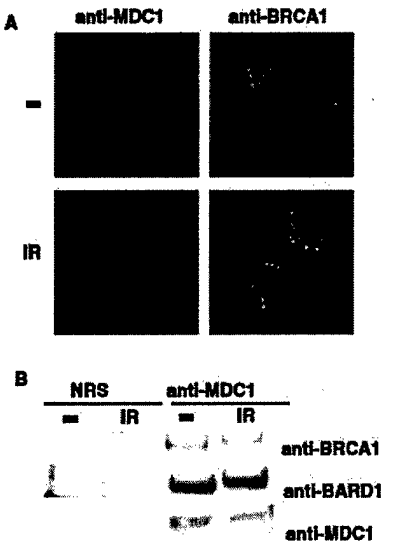

FIG. 2. **MDC1 interacts with BRCA1·BARD1.** *A*, HeLa cells were irradiated (10 Gy) or left untreated and 16 h later stained with anti-BRCA1 and anti-MDC1 antibodies as indicated. *B*, K562 cells were irradiated or left untreated, and MDC1 was then immunoprecipitated with normal rabbit serum (*NRS*) or anti-MDC1 antibodies. The immunoprecipitates were blotted with anti-BRCA1, anti-BARD1, and anti-MDC1 antibodies.

vivo (19), we further determined whether MDC1 could also associate with BARD1. As shown in Fig. 2*B*, similar to BRCA1, BARD1 also associates with MDC1. These results suggest that MDC1 interacts with the BRCA1·BARD1 complex and may be involved in a BRCA1-mediated genome maintenance function.

The interaction between MDC1 and BRCA1 led us to investigate the relationship between MDC1 and BRCA1. We used siRNA technology to down-regulate either BRCA1 or MDC1. Transfection of HeLa cells with BRCA1 siRNA or MDC1 siRNA resulted in the depletion of BRCA1 and MDC1, respectively (Figs. 3, *A* and *B*, and 4*A*). As shown in Fig. 3*A*, depletion of MDC1 by siRNA transfection led to a complete loss of BRCA1

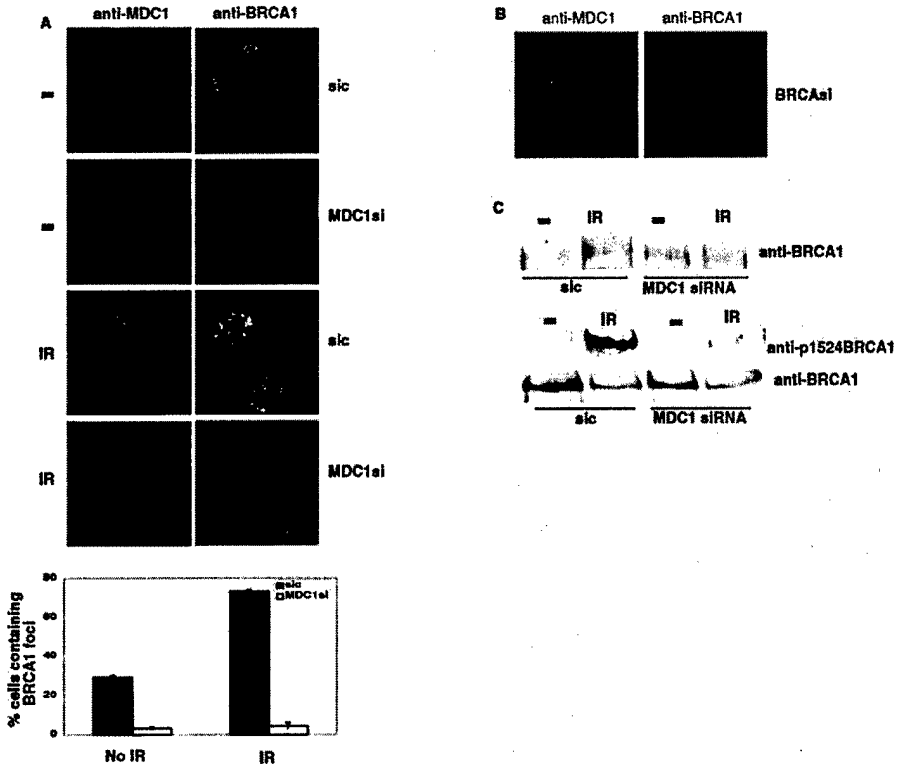

FIG. 3. **MDC1 regulates BRCA1 foci formation and phosphorylation.** *A*, HeLa cells were transfected with control siRNA or MDC1 siRNA, irradiated (10 Gy) or left untreated, and then stained with anti-BRCA1 and anti-MDC1 antibodies. Quantification of cells containing the indicated foci is shown in the *lower panel*. *B*, HeLa cells were transfected with control siRNA or BRCA1 siRNA. After 72 h, cells were irradiated (10 Gy) and 16 h later stained with anti-MDC1 and anti-BRCA1 antibodies as indicated. *C*, HeLa cells were transfected with control siRNA or MDC1 siRNA, irradiated (10 Gy) or left untreated, and then lysed and blotted with the indicated antibodies. *sic*, control siRNA; *MDC1si*, MDC1 siRNA.

foci in response to DNA damage (from 80% of cells containing BRCA1 foci in control samples to 4% in the MDC1 siRNA-transfected sample) (Fig. 3*A*). Only cells that retained MDC1 expression still formed BRCA1 foci (data not shown). Conversely, although transfection of HeLa cells with BRCA1 siRNA resulted in a loss of BRCA1 in 90% of the transfected cells (Fig. 3*B* and data not shown), the relocalization of MDC1 to nuclear foci following DNA damage was not affected by the depletion of BRCA1 (Fig. 3*B*). Furthermore the disappearance of BRCA1 foci in MDC1 siRNA-transfected cells is not due to a change in kinetics of BRCA1 foci formation since we could not detect BRCA1 foci either 6 h (data not shown) or 16 h after DNA damage (Fig. 3*A*). In unirradiated control cells, there are a subset of cells that contain BRCA1 foci (30%), which are the previously reported S-phase BRCA1 foci (12). Interestingly transfection of MDC1 siRNA in these cells led to a dramatic decrease of cells containing these BRCA1 S-phase foci (2% of cells containing BRCA1 foci) (Fig. 3*A*), suggesting that BRCA1 S-phase foci formation may also require MDC1. Transfection with a control siRNA, which was generated by introducing point mutations into MDC1 siRNA, had no effect on BRCA1 foci formation (Fig. 3*A*). Furthermore transfection with a second, different MDC1 siRNA (siRNA2) also abolished the localization of BRCA1 to nuclear foci (data not shown). Taken together these data strongly suggest that MDC1 is required for the recruitment of BRCA1 to the sites of DNA damage.

Using siRNA technology, we also found that down-regulation of H2AX significantly decreased IR-induced BRCA1 foci (data not shown). This is in agreement with early studies using H2AX-deficient mouse cells (20, 21). However, it is unlikely that MDC1 regulates BRCA1 foci formation solely through influencing γH2AX since MDC1 siRNA only partially reduced γH2AX foci (data not shown) but completely abolished BRCA1 foci in response to IR (Fig. 3*A*). In addition, while MDC1 regulated BRCA1 S-phase foci formation (Fig. 3*A*), H2AX does not seem to be required for the formation of BRCA1 S-phase foci (data not shown and see Ref. 20). Finally, while H2AX is

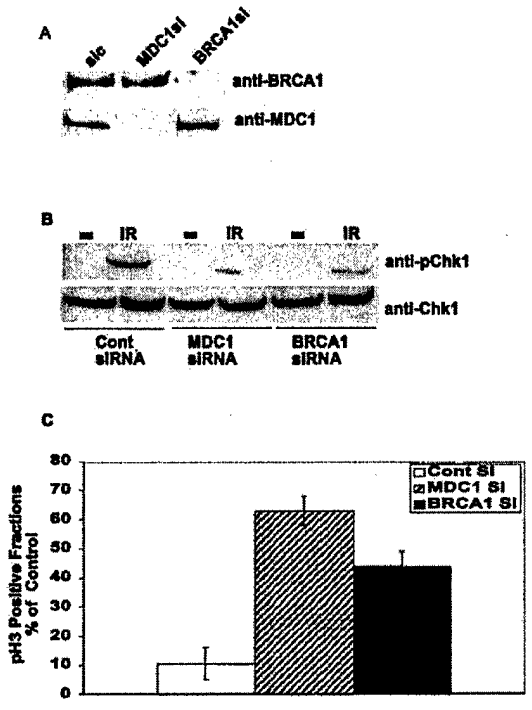

FIG. 4. **MDC1 regulates Chk1 activation and G_2/M checkpoint in response to DNA damage.** *A* and *B*, HeLa cells transfected with control siRNA, MDC1 siRNA, or BRCA1 siRNA were irradiated or left untreated, and cell lysates were blotted with the indicated antibodies. *C*, defective G_2/M checkpoint in cells transfected with MDC1 siRNA. HeLa cells transfected with control siRNA, MDC1 siRNA, or BRCA1 siRNA were irradiated (2 Gy) or left untreated. 1 h later cells were fixed and stained with anti-phospho-H3 antibodies, and phospho-H3-positive fractions were determined. The results were expressed as the percentage of the phospho-H3-positive fraction in irradiated cells compared with that in unirradiated control cells. *sic*, control siRNA; *MDC1si*, MDC1 siRNA; *BCRA1si*, BCRA1 siRNA; *Cont*, control; *Si*, siRNA; *pH3*, phospho-H3.

dispensable for DNA damage-induced hyperphosphorylation of BRCA1 (data not shown), MDC1 is required for BRCA1 phosphorylation following DNA damage (see below).

BRCA1 is phosphorylated by ATM at multiple residues including Ser-1387, Ser-1423, Ser-1457, and Ser-1524 after irradiation (22, 23). To further investigate the role of MDC1 in the regulation of BRCA1, we examined the phosphorylation of BRCA1 following DNA damage in MDC1-deficient cells. As shown in Fig. 3*C*, transfection with MDC1 siRNA resulted in defective hyperphosphorylation of BRCA1 following DNA damage as judged by its mobility shift by SDS-PAGE or by blotting with antibodies against phosphoserine 1524 of BRCA1. However, BRCA1 phosphorylation is normal in cells transfected with H2AX siRNA (data not shown). It is worth mentioning that the defective BRCA1 phosphorylation in MDC1 siRNA-transfected cells is only observed with low doses of irradiation (10 Gy or lower). When higher doses of irradiation are used (30 Gy), phosphorylation of BRCA1 observed in MDC1 siRNA-transfected cells is similar to that in control cells (data not shown), suggesting the possibility that an alternative pathway for BRCA1 phosphorylation is activated at higher doses. Recently 53BP1 has also been reported to regulate BRCA1 foci formation and phosphorylation (24). It is possible that MDC1 and 53BP1 might work together in regulating BRCA1 following DNA damage.

The defective BRCA1 localization and phosphorylation in MDC1 siRNA-transfected cells suggests that MDC1 might regulate BRCA1-mediated functions. It is well established that BRCA1 is involved in the maintenance of genome stability. BRCA1 participates in a number of activities following DNA damage including DNA repair (25), Chk1 activation (26), S-phase and G_2/M checkpoint control (27, 28), and cell survival (22). This prompted us to investigate how MDC1 affects BRCA1-mediated downstream events. Using MDC1 siRNA or BRCA1 siRNA, which specifically down-regulated MDC1 and BRCA1, respectively (Fig. 4*A*), we first examined the Chk1 activation following DNA damage. In response to DNA damage, Chk1 is phosphorylated at Ser-317 and Ser-345, and the phosphorylation of these sites is required for Chk1 activation (29). Down-regulation of either BRCA1 or MDC1 resulted in decreased Chk1 phosphorylation following DNA damage (Fig. 4*B*). BRCA1 has recently been shown to regulate the G_2/M checkpoint, probably through its regulation of Chk1 activation (26). Thus, we next examined the G_2/M checkpoint control in cells depleted of MDC1. As shown in Fig. 4*C*, HeLa cells transfected with control siRNA showed a marked decrease in mitotic fraction in response to γ irradiation. However, similar to cells transfected with BRCA1 siRNA, cells transfected with MDC1 siRNA showed significantly greater mitotic fraction after γ irradiation, suggesting a G_2/M checkpoint defect in cells lacking MDC1 or BRCA1. Taken together these results suggest that MDC1 is involved in the G_2/M checkpoint, probably through regulating the BRCA1-Chk1 pathway.

Emerging evidence suggests that MDC1 is an important mediator of DNA damage responses. In addition to its role in BRCA1 foci formation and phosphorylation, MDC1 also interacts with activated Chk2 and regulates S-phase checkpoint, p53 stabilization, and radiation-induced apoptosis (7). A recent report also links MDC1 to the Chk2 pathway (11). Furthermore we and others have shown that MDC1 regulates NBS1 foci formation.[3,5] These findings suggest that MDC1 regulates multiple DNA damage signaling pathways. MDC1 contains several protein-protein interaction domains including the FHA domain, BRCT domain, and internal repeated sequences. It is plausible that MDC1 acts as an adaptor protein to recruit downstream signaling molecules to the sites of DNA damage in a role similar to that of Grb2 or Shc in receptor tyrosine kinase-mediated signaling pathways.

In summary, we have shown that MDC1 regulates BRCA1 foci formation, phosphorylation, and G_2/M checkpoint control in response to DNA damage. These results establish a critical role of MDC1 in the regulation of DNA damage checkpoint. Given that dysregulation of DNA damage checkpoints (*e.g.* mutations of p53 and BRCA1) frequently leads to tumorigenesis, one may wonder whether MDC1 functions as a tumor suppressor. Future genetic studies of MDC1 will be conducted to test this possibility.

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